

Attorney Docket No.: 48544.00012 (DC-0293)
Inventor: Paula Sundstrom
Serial No.: 10/672,074
Filing Date: September 29, 2003
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Amendments to the Specification:

Please replace the paragraph [0001] beginning at page 1 with the following rewritten paragraph:

--[0001] This application is a continuation-in-part of application Serial No. 09/801,774, now U.S. Patent No. 6,706,688, filed 9 March 2001, for Methods for Regulating Bud-Hypha ~~Transitions~~ Transitions and cAMP Levels by the Adenylate ~~Cyclase-Associates~~ Cyclase-Associated Protein Gene, *CAP1*, which is expressly incorporated fully herein by reference.--

Please replace the paragraph [0015] beginning at page 7 with the following rewritten paragraph:

--[0015] The *C. albicans* PDE1 gene was previously cloned and found to complement heat-shock sensitivity of *S. ~~cervisiae~~ cerevisiae* pde2 mutants, but did not affect morphogenesis (Hoyer et al., 140 MICROBIOLOGY 1533-1542 (1994)). The present invention illustrates that disruption of *PDE2* activates the cAMP-signaling pathway by limiting the ability to degrade cAMP in *C. albicans*, whereas overexpression down-regulates the cAMP-signaling pathway in *C. albicans*. The present invention is the first to show that a pde2/pde2 mutant may be hyperactive in forming germ tubes and production of HWP1, which may be accompanied by attenuated virulence. Further, the present invention is the first to describe genetic evidence showing that cAMP promotes true hyphae formation in *C. albicans*. The present invention also describes interference with *CAP1* function, which has potential for providing novel strategies for interfering with candidiasis.--

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Please replace the paragraphs [0018]-[0019] beginning at page 7 with the following rewritten paragraphs:

[0018] The present invention relates to methods for altering the expression of one or more genes associated with the regulation of the cAMP-PKA signaling pathway in *C. ~~albicans~~ albicans*, resulting in the interference of the virulence properties and disrupting the morphological transitions of *C. albicans*. In particular, virulence properties may include, but are not limited to, adhesive properties, invasive properties (e.g., ability to degrade extracellular matrix proteins and ability to block neutrophil oxygen radical production and degranulation) and proliferative properties. Additionally, the disruption of the morphological transitions comprises the transition from the budding form to the hyphal growth form.

[0019] In a further embodiment, the genes associated with the cAMP-PKA pathway comprise the *C. albicans* *PDE2* gene and the *C. albicans* homologue of the *CAP1* gene. In a yet further embodiment of the present invention, expression of the *PDE2* gene may be disrupted by interfering with *PDE2* transcription mediated by *cis* acting sequences. The *cis* acting sequences of the present invention ~~comprises~~ comprise *cis*-regulatory elements, such as upstream ~~activating~~ activating sequences (UAS) and upstream regulatory sequences. In a particular embodiment of the present invention, the *cis*-regulatory element comprises a cAMP response element (CRE) located in the promoter region of the *PDE2* gene. In an alternate embodiment, *PDE2* gene expression may be disrupted by interfering with DNA binding proteins (BP) that bind to *PDE2* *cis*-regulatory elements. In particular, the DNA BP comprises the CRE

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binding protein. Disruption of *PDE2* gene expression may lead to enhanced activation of the cAMP-PKA signaling pathway, hyperactive germ tube formation, avirulence, attenuated avirulence, hyperactive production of *HWP1*, sensitivity to nutrient starvation, defective entry into stationary phase, and/or increased sensitivity to exogenous cAMP in *C. albicans*.--

Please replace the paragraph [0026] beginning at page 9 with the following rewritten paragraph:

--[0026] ~~FIGURE 3 depicts~~ FIGURES 3A-3B depict the primary structure alignment of *C. albicans* Cap1 with CAPs of other organisms. Multiple sequence alignments of CAPs from *C. albicans* (CaCAP1; SEQ ID NO:1), *S. cerevisiae* (ScCAP; SEQ ID NO:16), *S. pombe* (SpCAP; SEQ ID NO:17), Mouse (MouseCAP1; SEQ ID NO:18), and Human (HumanCAP1; SEQ ID NO:19) were performed with ClustalW (Thompson et al., 22 NUCL. ACIDS RES. 4673-80 (1994)) and illustrated with ~~MacVector~~ MACVECTOR® 6.5.3 (Oxford Molecular Company). Solid lines indicate residues for the conserved RLE/RLE motif (Hazen et al., 24 INFECT. IMMUN. 661-6 (1979); Hood et al., 28 CLIN. INFECT. DIS. 587-96 (1999); Huang et al., 30 SCAND. J. INFECT. DIS. 137-42 (1998); Kawamukai et al., supra; Kawarabayashi et al., 28 GYNECOL. OBSTET. INVEST. 132-7 (1989); Kimura and Pearsall, 21 INFECT. IMMUN. 64-8 (1978); Kohler and Fink, 93 PROC. NATL. ACAD. SCI. USA 13223-8 (1996); Kurtz et al., 6 MOL. CELL. BIOL. 142-9 (1986); Kyte and Doolittle, 157 J. MOL. BIOL. 105-32 (1982); Lebrerer et al., 7 CURR. BIOL. 539-46

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(1997)), the polyproline region (289-297) and two consensus SH3-binding motifs (358-361 and 364-367) *C. albicans*.--

Please replace the paragraph [0036] beginning at page 12 with the following rewritten paragraph:

--[0036] FIGURE 13 depicts the highly conserved phosphodiesterase signature sequence of Pde2 of *C. albicans*, *S. cerevisiae* and humans (CaPDE2 (SEQ ID NO. ~~3~~ NO:3), ScPDE2 (SEQ ID NO. ~~4~~ NO:4) and HuPDE2A3 (SEQ ID NO. ~~5~~ NO:5), respectively). The alignment was created with ClustalW (Thompson et al., 22 NUCL. ACIDS RES. 4673-4680 (1994)) and illustrated with ~~MacVector~~ MACVECTOR® 6.5.3 (Oxford Molecular Company, Burlington, Mass.). Conserved phosphodiesterase signature motif was found using the ProDom database ~~located on the World Wide Web at~~ ~~toulouse.inra.fr/prodom/doc/prodom.html~~.--

Please replace the paragraph [0116] beginning at page 35 with the following rewritten paragraph:

--[0116] An embodiment of the present invention relates to various methods for altering the expression of one or more genes associated with the regulation of the cAMP-PKA signaling pathway in *C. ~~albicans~~ albicans*, resulting in the interference of the virulence properties and disrupting the morphological transitions of *C. albicans*. In particular, the *C. albicans* virulence properties may include, but are not limited to, adhesive properties, invasive properties (e.g., ability to degrade extracellular matrix proteins and ability to block neutrophil oxygen radical production and degranulation) and proliferative

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properties. Additionally, the disruption of *C. albicans* morphological transitions comprises the transition from the budding form to the hyphal growth form.--

Please replace the paragraph [0224] beginning at page 70 with the following rewritten paragraph:

--[0224] CAI4 (*CAP1/CAP1, ura3/ura3*) was transformed using spheroplast transformation (Kurtz et al., 6 MOL. CELL. BIOL. 142-9 (1986)) with 10 µg of pCAPURA3 digested with *Pst*I to release the *CAP1* disruption cassette. *Ura*⁺ transformants with an *CAP1/cap1::hisG-URA3-hisG* genotype were identified by Southern blotting using *Hind*III-digested genomic DNA (Scherer et al., 25 J. CLIN. MICROBIOL. 675-9 (1987)). Southern blots were probed with *hisG-URA3-hisG* from p5921 and PCR-1.2 (FIGURE 2A). PCR-1.2 (nucleotides 98 to 1318) was generated by PCR using pGHCP17 as template and oligonucleotides CAP-R4 (5'CCATTTTCCAAGAGGAAGCA3'; SEQ ID NO:20) and CAP-F4 (5'CCGACACTGCATTTGCTTTA3'; SEQ ID NO:21). Probes were labeled using the enhanced chemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection System (Amersham). CAC1-1 (*ura3/ura3 CAP1/cap1::hisG*) was selected on YNB media (0.002% uridine) containing 0.05% 5-fluoroorotic acid (5-FOA) (Boeke et al., 197 MOL. GEN. GENET. 345-6 (1984)) and used in a second round of transformation to disrupt the remaining copy of *CAP1*. Colony PCR (van Zeijl et al., 59 J. BIOTECH. 221-4 (1997)), using the ~~TaqPlus~~ TaqPlus® Long PCR system (Stratagene) with primers CAP-R4 and CAP-F4, and Southern blotting were used to determine genotypes. Gene inactivation was confirmed by Northern blot analysis and RT-PCR.--

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Please replace the paragraphs [0227]-[0028] beginning at page 71 with the following rewritten paragraphs:

--[0227] Total RNA was isolated (Staab et al., 271 J. BIOL. CHEM. 6298-305 (1996)) from middle logarithmic phase yeast cultured in 250 ml YNB at 27°C or germ tubes (yeasts for the *cap1/cap1* mutant) cultured for three hours in M199 at 37°C and treated with RNase-free DNase I (~~Gibo-BRL~~ GIBCO-BRL). Probes were PCR-1.2 (FIGURE 2A) and a 687 bp PCR product amplified from the 18S rRNA gene of *C. albicans* SC5314 (Makimura et al., 40 J. MED. MICROBIOL. 358-64 (1994)) using primers (5'-ACTTTCGATGGTAGGATAG-3', SEQ ID NO:22; and 5'-TGATCATCTTCGATCCCCTA-3', SEQ ID NO:23). Electrophoresis, radiolabeling of probes using the random primer method (Feinberg and Vogelstein, 132 ANAL. BIOCHEM. 6-13 (1983); Feinberg and Vogelstein 137 (Addendum) ANAL. BIOCHEM. 266-7 (1984)), hybridization and molecular size determination were performed as described by Staab et al. (J. BIOL. CHEM., *supra*), except that blots were hybridized first with the *CAP1* probe (10⁷ cpm), autoradiographed and then used with the 18S rRNA probe (10⁶ cpm).

Example 5: RT-PCR

[0228] The first-strand cDNA was synthesized using 1 µg of total RNA according to the manufacturer's directions (Promega; Reverse Transcription System) and was diluted in a final 100 µl volume of nuclease-free water. Two PCR products representing the 5'-(1 to 605) and 3'-(922 to 1634) portions of *CAP1* message (FIGURE 2A) were amplified from the first-strand cDNA (10 µl) using oligonucleotides CAP-NRT1 (5'-ATGTCAACCGAGGAGAGTCA-3'; SEQ ID NO:24) and CAP-F1 (5'-ATGTACGAGATTGGTGTAGG-3'; SEQ ID NO:25)

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and CAP-R3 (5'-AGTGAAAATCCATCTCCAGC-3'; SEQ ID NO:26) and CAP-3F1 (5'-CCAGCATGTTCAACAATTTGAG-3'; SEQ ID NO:27) respectively. ACT1 cDNA (304 bp), amplified using two ACT1-specific primers, ACT-3R (5'-GGAGTTGAAAGTGGTTTGGTCAATAC-3'; SEQ ID NO:28) and ACT-5L (5'-GGCTGGTAGAGACTTGACCAACCATTG-3'; SEQ ID NO:29) (Naglik et al., 67 INFECT. IMMUN. 2482-90 (1999)) served as a control. PCR products were detected by Southern blotting using PCR-1.6, which spanned the entire CAP1 coding region, as a probe (FIGURE 2A) PCR-1.6 (nucleotides 1 to 1634) was generated by PCR using pGHCP17 and oligonucleotides CAP-NRT1 and CAP-3F1. Probe PCR-1.6 was labeled with [α -³²P]-dCTP (Amersham) as for Northern blot except that 2×10^6 cpm was added to the membrane.--

Please replace the paragraph [0253] beginning at page 79 with the following rewritten paragraph:

--[0253] First strand synthesis is completed by adding isolated RNA and the appropriate primer. The primer/RNA mix is incubated followed by the addition of first strand reaction buffer, DTT, dNTPs, ~~RNasin~~ RNasin®, and ~~Superscript~~ Superscript™ II (~~Gibco-BRL~~ GIBCO-BRL) to the mix. Following a second incubation period, second strand synthesis buffer, dNTPs, DNA polymerase, RNase, DNA ligase, and RNase-free water are added. Following a third incubation period, DNA polymerase is added to each sample. Following a fourth incubation period, the cDNA is extracted and washed 3x with water in a column. After collection from the column, the cDNA is dried for *in vitro* transcription.--

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Please replace the paragraphs [0255]-[0256] beginning at page 79 with the following rewritten paragraphs:

--[0255] Amplified RNA (aRNA) from the first round amplification is mixed with random hexamers, incubated, chilled on ice, and then equilibrated at room temperature. For the initial reaction, first strand buffer, DTT, dNTPs, ~~RNasin~~ RNasin®, and reverse transcriptase are added to the aRNA mix, and then incubated. RNase is then added and the sample is incubated again. For second strand cDNA synthesis, primer is added to the aRNA reaction mix and the sample is incubated. Second strand synthesis buffer, dNTPs, DNA Polymerase, RNase, DNA ligase, and RNase-free water are added to the sample mix and the sample is incubated again. DNA polymerase is then added followed by sample incubation. The double-stranded cDNA is extracted to remove extraneous protein and purified to remove the unincorporated nucleotides and salts.

[0256] aRNA and random hexamers are mixed in a solution containing RNase-free water, heated, and then chilled on ice. For the labeling reaction, first strand buffer, DTT, ~~RNasin~~ RNasin®, d(GAT)TP, dCTP, labeled-dCTP, and reverse transcriptase are added to the aRNA mix and incubated at room temperature. The aRNA template is degraded and the sample incubated again at a suitable temperature. The probes are purified with ~~Microcon~~ Microcon® 30 Columns and ~~Qiagen~~ Qiagen® Nucleotide Removal Columns. The probes are vacuum-dried and resuspend in hybridization buffer.--